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Induction of vascularisation by an aqueous extract of the flowers of *Calendula officinalis* L. the European marigold

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Summary

Calendula officinalis L. (calendula) is a plant whose recorded history is indicative of intrinsic wound healing capabilities. The wound healing process involves several distinct phases in which the formation of new blood vessels plays an essential role. This report describes the angiogenic activity of a freeze-dried aqueous extract of the flowers of *Calendula officinalis* L. (the European marigold) utilizing the chick chorioallantoic membrane (CAM) assay. The CAM assay is a standard and well established method for assessing the angiogenic activity in impure and pure preparations and is suitable for studies requiring examination of large numbers of sample test materials. The angiogenic activity of calendula was measured by examination of CAMs using stereomicroscopy. Further histological investigation and quantification of neovascularization was performed utilizing microvascular counts. The histological sections of CAMs were also examined for the presence of hyaluronan (HA), a tissue glycosaminoglycan associated with neovascularization, by hyaluronidase digestion and staining of tissue sections by alcian blue. All calendula treated CAMs were positive for HA; no HA could be demonstrated in control CAMs. The numbers of microvessels in calendula-treated CAMs were statistically significantly higher than in the control CAMs ($p < 0.0001$). Thin layer chromatography indicated that the calendula extract contained water-soluble compounds such as flavonoids, but the exact nature of the active angiogenic component(s) has not yet been identified.

Key words: *Calendula officinalis* L., wound healing, flavonoids, hyaluronan, angiogenesis, CAM assays.

Introduction

Calendula officinalis L. (calendula) belongs to the Compositae or Asteraceae family, the tribe Calenduleae; and Genus Calendula L. comprising some 20 species. It is believed to have originated in Egypt and is primarily a Mediterranean genus, being widely grown as an ornamental plant and is naturalized elsewhere (Polunin, 1969; Norlindh, 1977). Calendula has bright yellow-to-orange flowerheads, the ligulate florets mistakenly called the flower petals have been used medicinally (British Herbal Pharmacopoeia, 1983). One of the earliest references to the medicinal use of calendula is from a 14th-century medical manuscript which refers to the "Golde" in the drawing out of evil humors. However familiarity with the use of calendula appeared to be widespread even in the 13th century (Arctander, 1960; Coats, 1968). These early manuscripts clearly identify the marigold used for its wound-healing capabilities as *Calendula officinalis* L. (Scheffer, 1979). This apparent tradition of calendula's assumed healing activity has persisted to the present day with abundant anecdotal and spurious evidence. It is frequently referred to in Materia Medicas and modern herbals without any real evidences to its efficacy in the treatment of human disorders (Palaiseul, 1977).

Intrinsic to earlier therapeutic claims of calendula are its ability to heal external open wounds, lacerations and ul-

cers, making acrid discharges healthy and free, and promoting the formation of granulation tissue (Kent, 1986; Boericke, 1987). Calendula has also been used to treat ulcerations of the stomach and bowel, hemorrhages, and acute and chronic skin lesions including burns (Palaiseul, 1977; Phatak, 1982). Other therapeutic uses are for gynecological, feverish, or toxic conditions; and as an antiseptic and topical anti-inflammatory agent (British Herbal Pharmacopoeia, 1983; Wren, 1988; Ody, 1983). By far, historical use of calendula is weighted in the area of wound healing and it is a constituent in many skin ointments (Lovell, 1993).

Some investigations have evaluated the chemistry of calendula in relation to therapeutic claims in the field of wound healing. A large number of these reports have come from Eastern Europe and provide minimal information. These studies tentatively corroborate calendula as being antiinflammatory, containing active triterpene glycosides (Schipochliev et al., 1981), and oxygen-containing terpene derivatives (Gracza, 1987). An antibacterial action has also been attributed to triterpene glycosides of calendula (Gracza and Szasz, 1968). Calendula has been reported to increase the rate of re-epithelialization, granulation tissue formation and the regeneration of dermal collagen in skin wounds (Klouček-Popova et al., 1982; Anonymous, 1983). The chemistry of calendula consists notably of pentacyclic triterpene alcohols, flavonoids such as quercetin, saponins and sesquiterpenes, and lactones like the calendins (Steinegger and Hänsel, 1968; Valadon Guy, 1977; Wilkomirski, 1985).

Exact information relevant to the purported wound healing role of any compound present in calendula is lacking. Preliminary indications are that calendula possesses anti-inflammatory activity related to triterpenoids and cholesterolic carotenoids (Fleischner, 1985; Gracza, 1987; Della Loggia et al., 1994), together with immunostimulating polysaccharides (Wagner et al., 1985). Calendula has also been listed in the European Union as a drug (Anonymous, 1994). The anti-inflammatory and antiviral activity of a related plant *Calendula arvensis* L. has also been reported (Chemli et al., 1990; De Tommasi et al., 1990; De Tommasi et al., 1991). The role of triterpenes and polysaccharides in inflammation is believed to be by intervention in immunological reactions, such as promotion of anti-inflammatory factors (Wagner, 1989). As mentioned earlier, adequate angiogenesis is a critical requirement of wound healing (Whalen and Zetter, 1992). This report describes the effect of a calendula extract on the neovascularization process.

Materials and methods

A. Preparation of freeze-dried extract of Calendula officinalis

Dried flowers of cultivated *Calendula officinalis* L. were supplied by Joseph Flach and Sons Ltd., Peterborough, Cambridgeshire, U. K. A voucher sample is kept at the Department of Biology, ICA Sciences, Manchester Metropolitan University, U. K. A freeze-dried extract was aseptically prepared by cold aqueous infusion of 100 g of dried calendula flowers into 1 l of sterile pyrogen-free distilled water (Fresenius Flowfusor Sterile Non-pyrogenic Solution, F. L. Manufacturing Ltd., Fresenius Health Care Group, Basingstoke England. U. K.). This was carried out overnight in a glass Pyrex beaker sealed with Parafilm-M (American Can Company, Merck Ltd., Laboratory Supplies, Magna Park, Lutterworth, Leicester, U. K.), and filtered through lead free glass wool (Fisons Scientific Equipment, Metlab Supplies Ltd., Hawarden, Clwyd, U. K.) to remove any remaining plant debris. The resultant solution was passed through grade 113 V folded filter paper (Whatman Labsales International Ltd., Maidstone, Kent, U. K.), producing a clear, dark-brown, sweet-smelling fluid. This was evenly divided into sterile amber 5 ml glass vials, immediately frozen (–20 °C) and freeze-dried. The vials were filled with gaseous

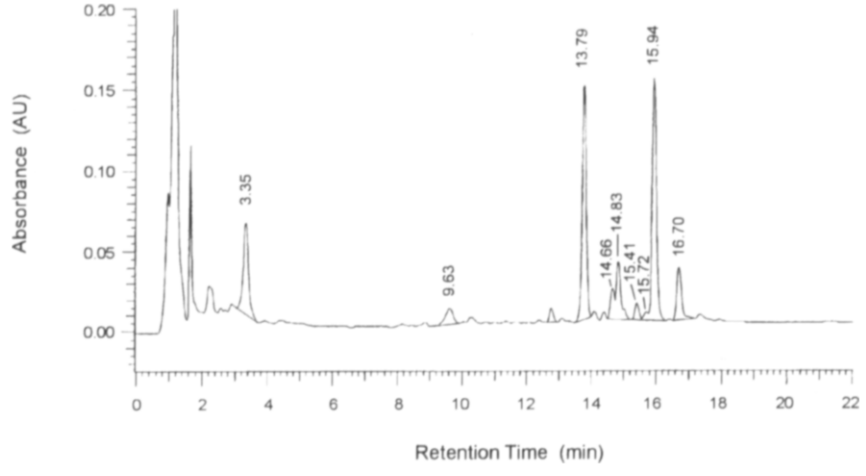

Fig. 7. HPLC-fingerprint – analysis of Calendula off. extract.

Conditions of HPLC-fingerprint analysis of Calendula off. extract

column:	Lichrospher 100 RP-18.5 µm
solvent:	A:H_2O/1% O, 1 n H_3PO_4
system:	B: acetonitrile/1% 0.1 n H_3PO_4
flow:	1,0 ml/min.
detector:	DAD
wavelength:	210 nm
gradient:	5–50% acetonitrile/1% 0.1 n H_3PO_4 in 30 min.
inj. Vol.:	10 µl (5,45 mg extract/ml MeOH)

nitrogen at room temperature (Linde Gas U.K. Ltd., Tunstall, Stoke-on-Trent, Staffordshire, U.K.) providing an inert environment for the samples, capped and sealed with metal crimp caps and rubber seal (Phase Separations Ltd., Deeside Industrial Park, Clwyd, U.K.) and stored at 2–8 °C.

B. The Chick chorioallantoic membrane (CAM) assay

The details of the CAM assay have been previously published (Kumar et al., 1984). Briefly, fertile chicken eggs were incubated at 37 °C for three days, then a portion of the eggshell was removed and the membranes allowed to collapse. The eggs were sealed with cellotape and returned to the incubator until 10 days old. A freeze-dried aqueous extract of *Calendula officinalis* flower heads was placed onto the surface of the CAM using a sterile spatula and lactose was applied to the control CAMs. The eggs were resealed and incubated for a further four days. After this period, 'blind' examination of the CAMs for any evidence of angiogenic stimulating activity was performed using a Wild M 3 Z Plan Zoom Stereomicroscope (Leica U.K. Ltd., Milton Keynes, U.K.). Active compounds induced new vessels to grow in towards the implantation site on the CAM, forming a 'spoked wheel' pattern of vessels radiating from the point of sample application. In negative CAMs, no neovascularization was observed. The CAMs were fixed *in situ* in 10% neutral buffered formalin (Merck Ltd., Laboratory Supplies, Magna Park, Lutterworth, Leicester U.K.), for 30 min before excision. The appropriate portions of the CAMs were excised for further investigation.

C. Histology

(I) General Staining

Formalin fixed treated and control CAMs were routinely processed for paraffin wax embedding (VIP 1000 Tissue processor, Bayer Diagnostics, Basingstoke, Hampshire, U.K.). CAMs were paraffin embedded, sectioned at 5 microns, mounted on glass slides and air dried at 37 °C for a minimum of 12 hours prior to staining. Sections were stained by Bayley's acid Giemsa for basic morphology and assessment of inflammatory cells (Drury and Wallington, 1980 a), and by alcian blue staining utilising *Streptomyces* hyaluronidase digestion (*Streptomyces hyalurolyticus*, purchased from, Sigma Chemical, Poole, Dorset, U.K.) for the demonstration of tissue hyaluronan (HA) (Brinkley and Morris-Wiman, 1987). The presence of HA was assessed as positive (Scored as 1+ to 5+) or negative.

(ii) Staining of microvessels, grading and counting

Microvascular density in tissue sections was assessed by modification of the method of Weidner et al. (1991) using a periodic acid-Schiff (PAS) method (Drury and Wallington, *1980 b)*, for demonstration of vascular basement membranes. Individual microvessel counts were made on 10 random microscopic fields of the CAM in the area of highest neovascularization using a Leitz Diaplan microscope (Leica Ltd., Milton Keynes, U.K.) at a magnification of x400 (0.1885 mm^2 per field). Each count was expressed as the mean microvessel count per CAM. Neovascularization of treated and untreated CAMs was compared using Student's t-test.

D. Thin Layer Chromatography

Thin layer chromatography (TLC) of a 3% aqueous extract of calendula was performed at room temperature by application of 30 microlitre spots using micro bulb pipettes onto silica gel 60 F 254 nm plates (Merck Ltd., Laboratory Supplies, Magna Park, Lutterworth, Leicester, U.K.). Separation of calendula was by ascending chromatography in a glass chamber utilizing butanol:acetic acid:water in the ratio 4:1:1 as a single-phase solvent. Chamber saturation prior to introduction of the plate was achieved by placing Whatman chromatography paper (Whatman Labsales International Ltd., Maidstone, Kent, U.K.), in the form of a *U* in the solvent, which had already been placed in the trough. The paper was moistened and then pressed against the sides of the chamber. The paper was then soaked again by tilting the chamber before introduction of the plate. The chamber was allowed to equilibrate for 1 hr at room temperature prior to TLC, using a solvent run of 12 cm. Plates were air dried at room temperature and examined by UV-lamp (emission maxima 254 and 366 nm). Spots present were graphically recorded using tracing paper and spray reagents respectively, viz modified anisaldehyde-sulphuric acid applied to the plates for visualisation of sugars, and with 10% antimony III chloride for flavonoids (Stahl, 1988).

In addition preliminary HPLC analysis was performed employing a reverse-phase system (Fig. 7).

Table 1. Assessment of angiogenic activity in *Calendula officinalis* L. extracts using the chick chorioallantoic membrane assay.

Source of CAM	*Nos. Positive* Nos. Tested	Hyaluronan in areas of neovascularisation
Calendula Treated	19/22	* Present in all treated CAMs
Control	0/9	Absent in all untreated CAMs

* The HA presence varied from weak to strong.

Table 2. Quantification of angiogenesis in *Calendula Officinalis* L. treated chick chorioallantoic membranes.

Source of CAM	Nos. of observations	Microvessel counts Mean ± S.E.	* Statistical analysis. Test vs. Control
Calendula treated	60	20.3 ± 2.9	$P < 0.0001$
Control	40	3.8 ± 0.2	

* Student's t test.

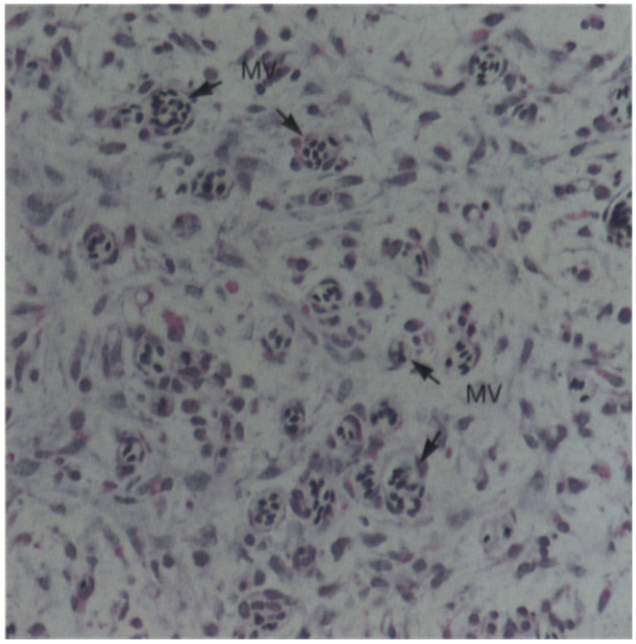

Fig. 1. Periodic acid-Schiff (PAS) method for demonstration of microvessels in tissue sections. *Calendula officinalis*-treated CAM showing high level of neovascularization. MV = microvessels, magnification x 400.

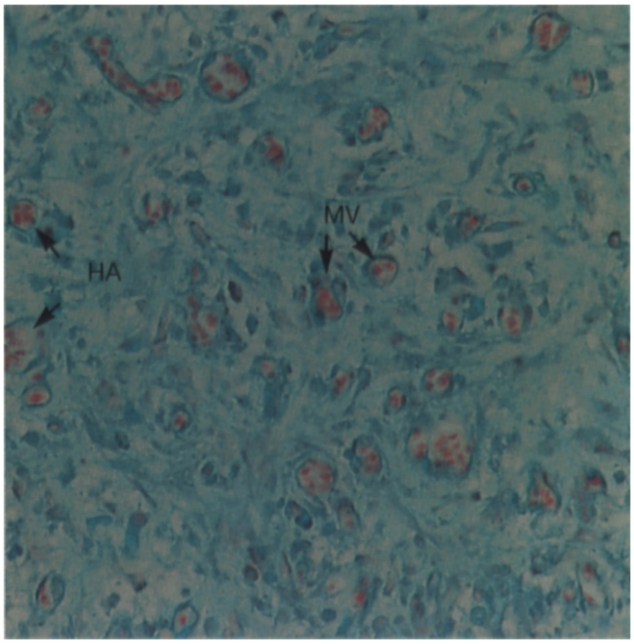

Fig. 2. Alcian blue stained tissue section for demonstration of tissue hyaluronan (HA). *Calendula officinalis* L. treated CAM showing increased HA deposition in an area of high neovascularization. HA = hyaluronan, MV = microvessels, magnification x 400.

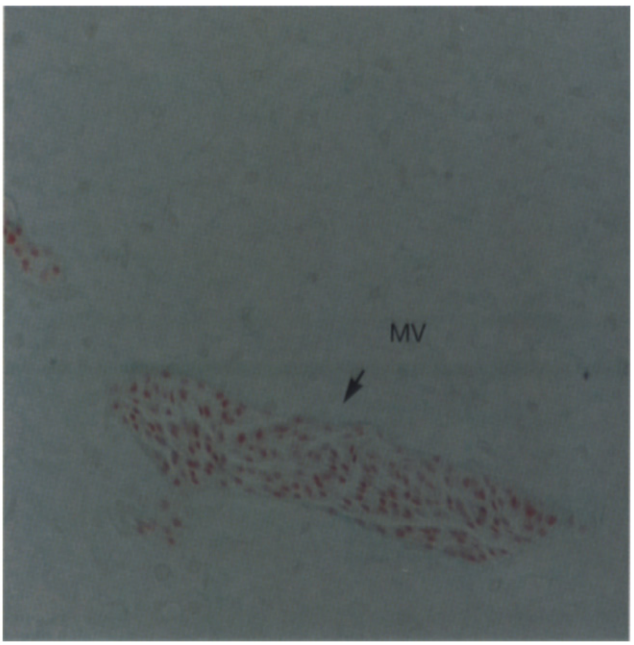

Fig. 3. Alcian blue stained tissue section of a control CAM, showing an absence of hyaluronan (HA) in an area of vascularization. BV = blood vessel, magnification x 400.

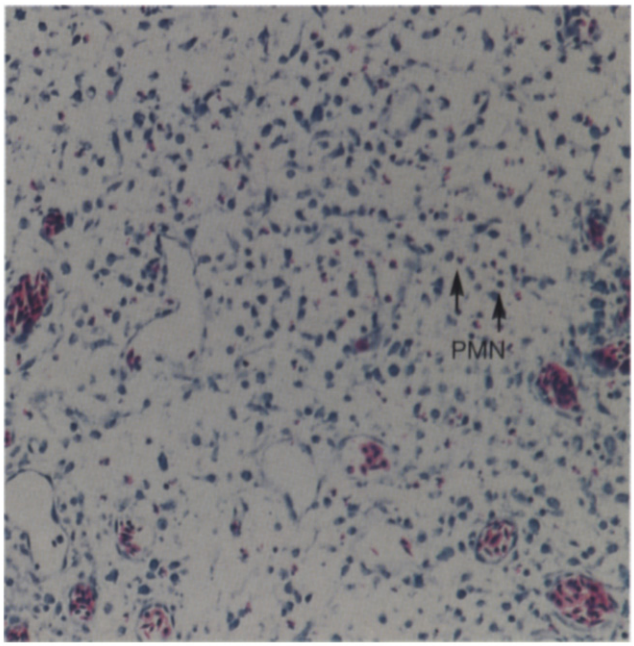

Fig. 4. Bayley's acid Giemsa-stained tissue section showing basic tissue morphology of a *Calendula officinalis*-treated CAM. PMNs = polymorphonuclear neutrophils, magnification x 400.

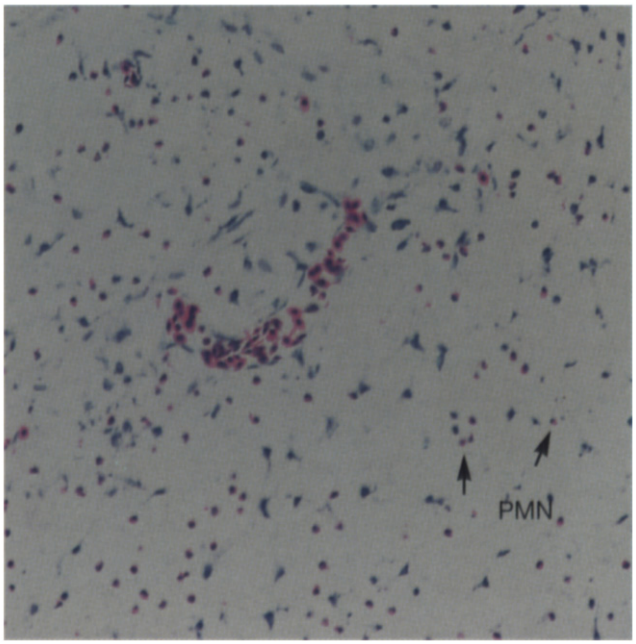

Fig. 5. Bayley's acid Giemsa-stained tissue section showing basic tissue morphology of a control CAM. PMNs = polymorphonuclear neutrophils, magnification x 400.

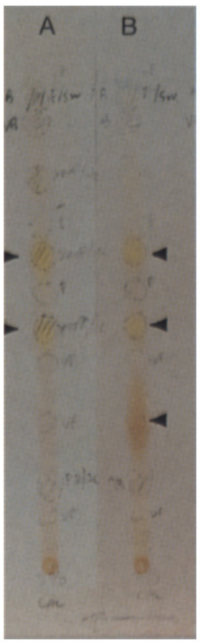

Fig. 6. Thin layer chromatogram of 3% aqueous extract of *Calendula officinalis*, on silica gel 60 F 254-nm plates, 10% antimony III chloride for visualisation of flavonoids. Flavonoid spots appeared bright yellow in daylight and had Rf values of 0.41 and 0.53 respectively (Track, A, ▶), the same spots were positive for sugars. The sugars were visualised by using modified anisaldehyde-sulphuric acid whereupon sugar the appeared green-black with a Rf value of 0.27, and pale green with Rf values of 0.41 and 0.53, repectively (Track, B, ▶).

Results

A method was standardized to obtain crude extracts of *Calendula officinalis* which were consistently highly angiogenic in the CAM assay (Table 1). The numbers of microvessels counted in tissue sections of calendula-treated CAMs were statistically significantly higher than in the control CAMs, $p < 0.0001$ (Table 2). All calendula treated CAMs were positive for HA; the level of positivity varied from weak to very strong. The high levels of HA deposition were in the same areas as those of high neovascularisation in calendula treated CAMs (Fig. 1 & 2). In contrast, the control CAMs were all negative for HA (Fig. 3). CAMs are sensitive to inflammatory agents, which can cause a spurious angiogenic response due to angiogenic factors being produced by inflammatory cells such as activated macrophages (Whalen and Zetter, 1992). The possibility of inflammatory cell angiogenic factors grossly influencing the assay was excluded by comparative assessment of Bayley's acid Giemsa stained tissue sections of both calendula treated CAMs and control CAMs. Calendula-treated CAMs and control CAMs had a similar morphological picture with small numbers of polymorphonuclear neutrophils (PMNs) in the intercellular matrix in both cases (Fig. 4 & 5). This indicated that the high levels of neovascularization observed in the treated CAMs could only be attributed to the effects of the calendula extract.

Qualitative analysis with TLC showed the calendula extract to be a complex mixture mainly comprised of water soluble flavonoid and sugar moieties. Flavonoid moieties in the calendula extract appeared in daylight as pale yellow to yellow spots on TLC plates, which after spraying with 10% antimony III chloride appeared bright yellow and gave a yellow fluorescence with long-wave (366 nm) UV light (Fig. 6, track A). These same spots were also found to contain sugar moieties that appeared pale green on TLC plates after spraying with modified anisaldehyde-sulfuric acid. A separate larger spot was also identified as containing a sugar moiety, which appeared green-black after spraying with modified anisaldehyde-sulfuric acid (Fig. 6, track B).

Discussion

The flavonoids comprise the largest group of naturally occurring, mainly water soluble plant phenols that are present both in the free state and as glycosides. Some 2,000 compounds have been identified and are plentiful in

the Compositae (Evans, 1989; Harborne, 1991). The role of flavonoids in plants are believed to provide a protective screen against the effects of ultra violet-B radiation, many of them are petal and fruit colorants. The color-dominant role of some flavonoids is also thought to be related to evolutionary insect pollination processes and to animal-dependent seed dispersal though not all flavonoids are colored (Hendry, 1993). Flavonoids are also important in growth control (Galston, 1969) and provide natural resistance to feeding by insects (Isman and Duffey, 1982). Generally, flowers are known to be rich sources of flavonoid compounds containing only small amounts of extraneous impurities: the yellow color imparted to plants has previously been attributed to polyhydroxy flavones and flavonols (Seshadri, 1962). Three flavonol glycosides have previously been isolated from the flowers of *Calendula officinalis* L.; these are quercetin-3 rutinoside, quercetin 3-glucoside and the 3-glucoside isorhamnetin (Valadon, 1977). Preparations containing flavonoids as principal biologically active ingredients have been used to treat a variety of conditions, including wound healing (Havsteen, 1983; Hanasaki et al., 1994), being anti-phlogistic (Moroney et al., 1988; Wagner, 1989; Panthong et al., 1994), and vasoprotective agents (Evans, 1989).

Hyaluronan (HA) is a major component of the extracellular matrix (ECM) and was first described in mammalian tissues in 1934 (Meyer and Palmer, 1934). Balazs et al. (1986) suggested that hyaluronic acid and hyaluronate should be reserved to specifically indicate the acid and salt forms of the polymer respectively, while the term 'hyaluronan' (HA) should be used for the polysaccharide in general terms irrespective of its degree of dissociation. HA is a negatively charged, high molecular weight glycosaminoglycan (GAG) consisting of repeating disaccharide units of *N* acetylglucosamine and glucuronic acid. It differs from other GAGs in its lack of sulphation and absence of covalently linked protein. HA has been reported to have a role in embryogenesis, tissue healing, and numerous pathological states in adult life. For instance, during embryonic differentiation and wound healing HA levels are initially high and then decrease rapidly in response to an increase in hyaluronase (HAase). Furthermore, the molecular size of HA fragments can vary during the course of embryogenesis and tissue repair (Kumar et al., 1984).

Regarding the role of HA in angiogenesis, under pathological conditions associated with abnormal angiogenesis, such as tumors, diabetic retinopathy and rheumatoid arthritis, neovascularization occurs adjacent to a hyaluronate-rich fluid or stroma (Rooney and Kumar, 1996). Elevated levels of circulating HA are seen in sera of patients with cancer, rheumatoid arthritis and psoriasis. This suggests that freely diffusable and degraded HA is produced at these sites of vessel formation (Rooney et al., 1995). This is supported by observations that tissue-cultured rheumatoid synovial cells and activated synovial fibroblasts synthesize increased amounts of HA that have a much lower molecular weight than that produced by normal cells (Kumar et al., 1994). Histochemical studies have demonstrated that the GAGs of mature vessels differ from those of newly formed capillary sprouts (Rooney and Kumar, 1996). At the migrating tip of a capillary sprout, the basal lamina contains mainly HA, which perhaps aids endothelial cell migration and serves as a substratum for the mature lamina. It has been found that the size-dependent effects of HA are mediated through a cell surface receptor and may require endocytosis of the HA (West and Kumar, 1989).

Our observations of the occurrence of high levels of HA deposition in areas of neovascularisation in the CAM are consistent with the work of Ausprunk (1986) and Elenius et al. (1991), in which HA has been shown to play a role in the formation, alignment and migration of newly formed capillaries. As mentioned earlier, HA has also been implicated in a number of biological processes (Fraser and Laurent, 1989; Toole et al., 1989; Kumar et al., 1994), including the control of osmoregulation, interaction with proteins via sieve and exclusion effects, as tissue lubricants and for stabilization of tissue structures (Comper and Laurent, 1978). High concentrations of HA have also been noted in many tissues undergoing remodelling and morphogenesis (Toole et al., 1991). Its synthesis has been implicated in cellular processes important in wound healing, such as cell locomotion and proliferation (Brecht et al., 1986; Turley, 1992). Previous studies have also shown that HA oligosaccharides can induce endothelial cell proliferation and migration *in vitro*, and angiogenesis *in vivo* (Rooney et al., 1995). The findings of this study suggests that calendula contains water soluble flavonoids and or other compounds capable of inducing HA deposition and increasing the rate of neovascularization, thus lending credence to its wound-healing potential. The precise nature of the angiogenic moiety in the calendula extract has not been determined. From our qualitative TLC data on the standardised crude *Calendula* extract, it has been shown that the predominant chemical moieties present in the extract have been identified as flavonoids and flavonoid glycosides. Further work is currently being undertaken in order to identify the exact chemical moietie(s) responsible for the angiogenic activity using HPLC fingerprinting and mass spectroscopy. Flavonoids are known to inhibit lysosomal hydrolases that degrade glycosaminoglycans (Brown, 1980) and have been reported to inhibit hyaluronidase activity (Kuppusamy et al., 1990). It may be that the increased presence of HA noted by us in the *Calendula*-treated CAMs was induced by the inhibition of hyaluronidase by flavonoids.

References

Anonymous: Lipoid extracts of Calendula blossoms [editorial]. *Cosmetics and Toiletries 98:* 75, 1983.

Anonymous: Report on the 3rd International ESCOP-Symposium [editorial]. *Phytomedicine 1:* 173–176, 1994.

Arctander, S.: Perfume and Flavor Materials of Natural Origin. Det Hoffensbergske Etablissement, Elizabeth, N.J., p. 399, 1960.

Ausprunk, D.H.: Distribution of hyaluronic acid and sulfated glycosaminoglycans during blood-vessel development in the chick chorioallantoic membrane. *Amer. J. Anat. 177:* 313–331, 1986.

Balazs, E.A., Laurent, T.C., Jeanloz, R.W.: Nomenclature of hyaluronic acid. *Biochem. J. 235:* 903, 1986.

Boericke, W.: Materia Medica With Repertory. 9th ed. B. Jain Publishers Ltd PVT, New Delhi, p. 156, 1987.

Brecht, M., Mayer, U., Schlosser, E., Prehm, P.: Increased hyaluronate synthesis is required for fibroblast detachment and mitosis. *Biochem. J. 239:* 445–450, 1986.

Brinkley, L.L., Morris-Wiman, J.: Computer-assisted analysis of hyaluronate distribution during morphogenesis of the mouse secondary palate. *Development 100:* 629–635, 1987.

British Herbal Pharmacopoeia: British Herbal Medicine Association Scientific committee, Bournemouth U.K., pp. 44–45, 1983, third impression 1989.

Brown, J.P.: A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. *Mutation Research 75:* 243–277, 1980.

Chemli, R., Toumi, A., Queslati, S., Zouaghi, H., Boukef, K., Balansard, G.: *Calendula arvensis* L. Impact des saponines sur la toxicité, le pouvoir hémolytique et l'activité anti-inflammatoire. *J. Pharm. Belg. 45:* 12–16, 1990.

Coats, A.M.: Border Flowers. In: Flowers And Their Histories. Adam & Charles Black, London, pp. 35–37, 1956, reprint 1968.

Comper, W.D. and Laurent, T.C.: Physiological function of connective tissue polysaccharides. *Physiol. Rev. 58:* 255–315, 1978.

Della Loggia, R., Tubaro, A., Sosa, S., Becker, H., Saar, St., Isaac, O.: The Role of Triterpenoids in the topical anti-inflammatory activity of *Calendula officinalis* Flowers. *Planta Med. 60 (6):* 516–520, 1994.

De Tomassi, N., Pizza, C., Conti, C., Orsi, N., Stein, L.M.: Structure and in vitro antiviral activity of sesquiterpene glycosides from *Calendula Arvensis. J. of Natural Prod. 53 (4):* 830–835, 1990.

De Tomassi, N., Conti, C., Stein, L.M., Pizza, C.: Structure and *in vitro* antiviral activity of triterpenoid saponins from *Calendula Arvensis. Planta Med. 57:* 250–253, 1991.

Drury, R.A.B., Wallington, E.A.: Bone and decalcification. In: Carleton's Histological Technique. 5th ed. Oxford University Press, Oxford–New York–Toronto, p. 216, 1980 a.

Drury, R.A.B., Wallington, E.A.: Methods for Carbohydrates, Polysaccharides, and Mucosubstances. In: Carleton's Histological Technique. 5th ed. Oxford University Press, Oxford–New York–Toronto, pp. 237–240, 1980 b.

Elenius, K., Vainio, S., Laato, M., Salmivirta, M., Thesleff, Jalkanen, M.: Induced expression of syndecan in healing wounds. *J. Cell Biol. 114:* 585–595, 1991.

Evans, W.C.: Phenols And Phenolic Glycosides. In: Trease and Evans' Pharmacognosy, 13th ed. Baillière Tindall, London–Philadelphia–Toronto–Sydney–Tokyo, pp. 419–423, 1989.

Fleischner, A.M.: Plant extracts: To accelerate healing and reduce inflammation. *Cosmetics and Toiletries 100:* 45–58, 1985.

Fraser, J.R.E., Laurent, T.C.: Turnover and metabolism of hyaluronan. In: The Biology of Hyaluronan, Ciba Foundation Symposium 143. A Wiley-Interscience Publication. John Wiley & Sons, Chicester–New York–Brisbane-Toronto-Singapore, pp. 41–59, 1989.

Galston, A.W.: Flavonoids and Photomorphogenesis in Peas. In: Perspectives in Phytochemistry, Proceedings of the Phytochemical Society Symposium. (Eds. J.B. Harborne and T. Swain) Academic Press, London and New York, pp. 193–204, 1969.

Gracza, L. and Szasz, K.: Examination of active agents of petals of marygold (*Calendula officinalis* L.) *Acta Pharm. Hung. 38:* 118–125, 1968.

Gracza, L.: Oxygen-Containing Terpene Derivatives from *Calendula Officinalis. Planta Med. 53:* 227, 1987.

Hanasaki, Y., Ogawa, S., Fukui, S.: The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radical Biology And Medicine 16 (6):* 845–850, 1994.

Harborne, J.B.: Phytochemical Methods. 2nd ed. Chapman Hall, London–New York–Tokyo–Melbourne–Madras, p. 55, 1991.

Havsteen, B.: Flavonoids, a class of natural products of high pharmacological potency. *Biochemical Pharmacology 32 (7):* 1141–1148, 1983.

Hendry, G.A.F.: Plant Pigments. In: Plant Biochemistry and Molecular Biology. (Eds. P.J. Lea and R.C. Leegood) John Wiley and Sons, Chichester–New York–Brisbane–Toronto–Singapore, pp. 190–193, 1993.

Isman, M.B., Duffey, S.S.: Toxicity of tomato phenolic compounds to the fruitworm. *Heliothis Zea. Ent. Exp. & Appl. 31:* 370–376, 1982.

Kent, J.T.: Lectures on Homœpathic Materia Medica. B. Jain Publishers (P) Ltd, New Delhi, p. 354, 1986.

Klouček-Popova, E., Popov, A., Pavlova, N., Krusteva, S.: Influence of the physiological regeneration and epithelization using fractions isolated from *Calendula Officinalis. Acta Physiol. Pharmacol. Bulg. 8 (4):* 63–67, 1982.

Kumar, S., Keegan, A., Erroi, A., West, P., Gaffney, J.: Response of tissue cultured endothelial cells to angiogenesis factors. *Prog. Appl. Microcirc. 4:* 54–75, 1984.

Kumar, S., Ponting, J., Wang, M.: Hyaluronan and angiogenesis: Molecular mechanisms and clinical applications. In: Angiogenesis, Molecular Biology, Clinical Aspects. NATO ASI series A: Life sciences, vol. 263. (Eds. M.E. Maragoudakis, P.M. Gullino, P.I. Lelkes) Plenum Press, New York and London, pp. 219–241, 1994.

Kuppusamy, U.R., Khod, H.E., Das, N.P.: Structure-activity of flavonoids as inhibitors of hyaluronidase. *Biochemical Pharmacology 40:* 397–401, 1990.

Lovell, C.R.: Plants and the Skin. Blackwell Scientific Publications, London–Edinburgh–Boston–Melbourne–Paris–Berlin–Vienna, p. 156, 1993.

Meyer, K., Palmer, J.W.: The polysaccharide of the vitreous humor. *J. Biol. Chem. 107:* 629–634, 1934.

Moroney, M.-A., Alcaraz, M.J., Forder, R.A., Carey, F., Hoult, J.R.S.: Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an antiinflammatory flavonoid glycoside and related aglycone flavonoids. *J. Pharm. Pharmacol. 40:* 787–792, 1988.

Norlindh, T.: Calendulae-systemic review. In: The Biology and Chemistry of the Compositae, Volume II. (Eds. V.H. Heywood, J.B. Harbourne, B.L. Turner) Academic Press, London–New York–San Francisco, pp. 961–987, 1977.

Ody, P.: The Herb Society's, Complete Medicinal Herbal. Dorling Kindersley Limited, London–New York–Stuttgart, p. 43, 1983.

Palaiseul, J.: The Green Guide To Health From Plants. Barrie & Jenkins Communica Europa, London, pp. 179–181, 1973, reprint 1977.

Panthong, A., Kanjanapothi, P., Tuntiwachwuttikul, P., Pancharoen, O., Reutrakul, V.: Anti-inflammatory activity of flavonoids. *Phytomedicine 1:* 141–144, 1994.

Phatak, S.R.: Materia Medica Of Homeopathic Medicines. Indian Books & Periodicals Syndicate, New Delhi, pp. 137–138, 1977, reprint 1982.

Polunin, O.: Flowers of Europe. Oxford University Press, London–New York–Toronto, pp. 451–452, 1969.

Rooney, P., Kumar, S., Ponting, J., Wang, J. M.: The role of hyaluronan in neovascularisation (Review). *Int. J. Cancer 60:* 632–636, 1995.

Rooney, P., Kumar, S.: The role of collagens and proteoglycans in tumour angiogenesis. In: Tumour Angiogenesis. Oxford University Press, Oxford (in press), 1996.

Scheffer, J. J. C.: De goudsbloem (*Calendula officinalis* L.) als geneeskruid in verleden en heden. *Pharm. Weekblad 114:* 1149–1157, 1979.

Schipochliev, T., Dimitriov, A., Aleksandrova, E.: Study on the antiinflammatory effect of a group of plant extracts. *Vet. Sci. 18 (6):* 87–94 (1981).

Seshadri, T. R.: Isolation Of Flavonoid Compounds From Plant Materials. In: The Chemistry of Flavonoid Compounds. (Ed. T. A. Geissman) Pergamon Press, Oxford–London–New York–Paris, pp. 6–33, 1962.

Stahl, E.: Thin-Layer Chromatography. 2nd rev. ed. Springer-Verlag, Berlin–Heidelberg–New York, p. 857, 1969, reprint 1988.

Steinegger, E., Hänsel, R.: Lehrbuch der Pharmakognosie. 2nd rev. ed. Springer-Verlag, Berlin–Heidelberg–New York, pp. 195–197, 1968.

Toole, B. P., Munaim, S. I., Welles, S., Knudson, C. B.: Hyaluronate-cell interactions and growth factor regulation of hyaluronate synthesis during limb development. In: The Biology of Hyaluronan. Ciba Foundation Symposium 143. (Ed. E. D. Hay) John Wiley & Sons, Chicester–New York–Brisbane–Toronto–Singapore, pp. 138–149, 1989.

Toole, B. P.: Proteoglycans and Hyaluronan in Morphogenesis and Differentiation. In: Cell Biology of Extracellular Matrix 2nd Edition. Plenum Press, New York and London, pp. 305–341, 1991.

Turley, E. A.: Hyaluronan and cell locomotion. *Cancer Metast. Rev. 11:* 21–30, 1992.

Valadon Guy, L. R.: Arctoteae and Calenduleae-Chemical Review. In: The Biology and Chemistry of the Compositae, Volume II. (Eds. V. H. Heywood and J. B. Harbourne) Academic Press, London–New York–San Francisco, pp. 989–998, 1977.

Wagner, Von H., Proksch, A., Riess-Maurer, I., Vollmar, A., Odenthal, S., Stuppner, H. et al.: Immunstimulierend wirkende Polysaccharide (Heteroglykane) aus höheren Pflanzen. *Arzneim.-Forsch./Drug Res. 35 (7):* 1069–1075, 1985.

Wagner, H.: Search for new plant constituents with potential antiphlogistic and antiallergic activity. *Planta Medica 55:* 235–241, 1989.

Weidner, N., Semple, J. P., Welch, W. R., Folkman, J.: Tumour angiogenesis and metastasis-correlation in invasive breast carcinoma. *New Engl. J. Med. 324:* 1–8, 1991.

West, D. C., Kumar, S.: The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. *Exp. Cell. Res. 183:* 169–196, 1989.

Whalen, G. F. and Zetter, B. R.: Angiogenesis. In. Wound Healing: Biochemical & Clinical Aspects. 1st ed. (Eds. I. K. Cohen, R. F. Diegelmann, W. J. Lindblad) W. B. Saunders Company, Philadelphia–London–Toronto–Montreal–Sydney–Tokyo, pp. 77–95, 1992.

Wilkomirski, B.: Pentacyclic triterpene triols from *Calendula officinalis* flowers. *Phytochemistry 24: 12:* 3066–3067, 1985.

Wren, R. C.: Potter's New Cyclopaedia Of Botanical Drugs And Preparations. Revised ed. Saffron Walden, The C. W. Daniel Company Ltd., Essex, U. K., pp. 184–185, 1988.

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